Ex. 2

ANTIBODY CAPTURE ASSAYS—COMPARING ANTIBODY BINDING SITES USING AN ANTIBODY COMPETITION ASSAY

When antibody capture assays are performed using the antibody competition variation, the binding sites for two monoclonal antibodies can be compared. To use this assay, a sample of purified or partially purified antigen is bound to a solid support. Then two monoclonal antibodies are added, one labeled and one unlabeled. If the labeled antibody and the unlabeled antibody bind to separate and discrete sites on the antigen, the labeled antibody will bind to the same level whether or not the competing antibody is present. However, if the sites of interaction are identical or overlapping, the unlabeled antibody will compete, and the amount of labeled antibody bound to the antigen will be lowered. If the unlabeled antibody is present in excess, no labeled antibody will bind. With this assay, one monoclonal antibody will need to be purified and labeled, but none of the antibodies used as competitors need be purified.

Because only monoclonal antibodies are used in this technique, the antigen preparation need not be particularly pure. Samples containing as little as 1% pure antigen may be used, provided there are no cross-reacting proteins in the preparation.

1. Prior to the assay, purify and label each of the monoclonal antibodies that will be studied. Purification techniques are discussed in Chapter 8 and labeling techniques in Chapter 9.

2. The most widely used solid phase for this assay is a PVC microtiter plate. Cut the plate to the correct size for the number of assays.

3. Bind the standard antigen solution to the bottom of the wells by adding 50 μl of antigen solution to each well (20 μg/ml). PVC will bind approximately 100 ng/well (300 ng/cm^2). If maximal binding is required, use at least 1 μg/well. Although this is well above the capacity of the PVC, the binding will occur more rapidly, and the binding solution can be saved and used again. Avoid extraneous proteins, detergents, or other compounds that will lower the binding capacity of the PVC. If the choice of buffer is not dictated by experimental design, dilutions should be done in PBS.

   The amount of antigen bound to the wells should be high enough to produce an easily detectable antibody binding signal, but high concentrations also have some disadvantages. As the amount of antigen bound to the solid phase increases, the amount of competitor that is needed will increase. The amount of antigen should be titrated to the lowest needed to achieve a strong signal, thus increasing the sensitivity of the assay.

4. Incubate for 2 hr at room temperature in a humid atmosphere.

5. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient. The antigen solution or washes can be removed by flicking the plate over a suitable waste container.

6. The remaining sites for protein binding on the PVC plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr to overnight in a humid atmosphere at room temperature.

7. Wash the plate twice with PBS.

8. To each well add a mixture of two antibodies, one labeled and one unlabeled. Incubate for 2 hr at room temperature in a humid atmosphere.

   All antibody dilutions should be done in 3% BSA/PBS with 0.02% sodium azide. Buffers with azide should not be used when the detection system depends on horseradish peroxidase.

   To optimize this assay, the amount of labeled antibody should be titrated and used at a subsaturating level. These values can be determined in preliminary tests. For accurate quantitations, the amount of cold competitor should be titrated, and the midpoints of the competition curves compared.

9. Remove the unbound antibodies by four washes with PBS.

Determine and quantitate the amount of labeled antibody bound to the plate (p. 591).

NOTES

i. If the amount of antigen bound to the plate is too low to produce a strong signal, the solid support can be changed to nitrocellulose, which can bind approximately 1000 times more protein per surface area. Nitrocellulose can be purchased either as sheets or sealed to the bottom of microtiter wells. See p. 606 for special handling procedures.

ii. Any of blocking solutions on p. 496, Table 12.2, can be substituted for the 3% BSA solution.

iii. Antigen-coated plates can be stored after the blocking step (step 6) either in PBS with 0.02% sodium azide at 4°C for 1 week or indefinitely at −20°C after removing the blocking solution.

iv. For some applications or with some antibodies, the signal strength can be increased by using longer incubation times. For rapid semi-quantitative assays, the incubation times can be shortened to between 30 min and 1 hr. The blocking step must still be 2 hr or more.

MAKING THE ASSAY QUANTITATIVE

Competition between monoclonal antibodies for binding to an antigen can be quantitated in this assay. For this application, all monoclonal antibodies must be purified. A standard curve is established by titrating one monoclonal antibody against itself, that is, the same antibody is used for both the label and the competitor. The capacity of other unlabeled monoclonal antibodies to inhibit the binding of the labeled antibody to the plate is titrated. The results are plotted, and the concentrations necessary to achieve 50% inhibition of binding are compared.

OTHER APPLICATIONS

- **Screening hybridoma fusions for epitope-specific antibodies** This assay can be used to screen a hybridoma fusion for epitope-specific antibodies. The assay is best used as a complement to a preliminary screening procedure. In the preliminary screen, antibodies that bind to the antigen can be identified. Then, those monoclonal antibodies that bind antigen in a primary screen are tested in this antibody competition assay.

- **Quantitating antibodies specific for a particular epitope in polyclonal sera** If the competitor used in these assays is changed from an unlabeled monoclonal antibody to an unlabeled polyclonal antibody, the level of antibodies specific for that epitope in the polyclonal serum can be determined.

ANTIBODY CAPTURE ASSAYS—DETECTING AND QUANTITATING ANTIGENS USING ANTIBODY EXCESS ASSAYS

Using an antibody capture assay with the antigen competition variation, the presence and level of an antigen can be determined quickly. For this assay, purified or partially purified antigen is bound to the solid phase. A sample of the test solution, containing an unknown concentration of antigen, is added together with a labeled antibody specific for the antigen. Any antigen in the test solution will compete with the immobilized antigen for binding with the labeled antibody. After the unbound proteins are removed by washing, the assay is quantitated by the amount of labeled antibody that is bound to the solid phase. When high concentrations of antigen are found in the test solution, no labeled antibody will bind to the plate. When little or no antigen is present in the test solution, high amounts of labeled antibody will bind. To quantitate the levels of antigen, dilutions of the test solution are assayed. Comparing the titration curves for each solution will yield the relative levels of antigen.

The degree of purity needed for the immobilized antigen is determined by the specificity of the antibody. Polyclonal sera will contain extraneous antibodies that will bind and score against some impure antigen preparations. Often these can be distinguished by titrating the sera. Because of their specificity, solutions of monoclonal antibodies or affinity-purified polyclonal antibodies can be used with less pure sources of antigen.

One problem that may be encountered using this technique is that the labeled antibody may bind preferentially to the antigen on the plate. Quantitating this assay relies on a comparison of two avidities, the avidity of the antibody for the immobilized antigen and the avidity of the antibody for the antigen in solution. These avidities may not be the same. Because the antigen on the plate will be present in a high local concentration, the antibody may be able to bind bivalently to these antigens (see Chapter 3). These types of bivalent interactions cannot occur in solution when the antigen is monovalent. Not all immobilized antigens will promote bivalent binding, but many will. Problems generated by differences in avidity can be detected by comparing standard curves using known concentrations of antigens. If these assays give anomalous results, several steps can be used to help. First, try using lower concentrations of the antigen on the plate, thus decreasing the local concentration and decreasing the chances of bivalent binding. Second, premix the test solution with the labeled antibody and incubate for 30 min to 1 hr before adding to the plate. Third, the problem can be eliminated by using labeled Fab fragments (p. 626).

1. Prior to the assay, purify and label the primary antibody. The primary antibody can be labeled with iodine (p. 324), biotin (p. 340), or an enzyme (p. 342).

2. The most widely used solid phase for these assays is a polyvinylchloride (PVC) microtiter plate. Cut the plate to the correct size for the number of assays.

3. Prepare a standard solution of antigen. Avoid extraneous proteins and detergents.

4. Bind a sample of the standard antigen solution to the bottom of the wells by adding 50 μl of antigen solution to each well. PVC will bind approximately 100 ng/well (300 ng/cm^2). If maximal binding is required, use at least 1 μg/well (20 μg/ml). Although this is well above the capacity of the PVC, the binding will occur more rapidly, and the binding solution can be saved and used again. If the antigen is rare or expensive, lower concentrations can be used. Unless another buffer is dictated by experimental conditions, dilutions can be done in PBS.

   To optimize the assay, the amount of antigen on the plate should be adjusted to a level that will just bind all of the input labeled antibody but still give a satisfactory signal strength. See p. 573.

5. Incubate for 2 hr at room temperature in a humid atmosphere.

6. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient. The antigen solution or washes can be removed by flicking the plate over a suitable waste container.

7. The remaining sites for protein binding on the PVC plate must be saturated by incubating with blocking buffer. Fill the wells to the top with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr to overnight in a humid atmosphere at room temperature.

8. Wash the plates twice with PBS.

9. Add 50 μl of the antigen test solution together with the labeled antibody to each well. Incubate for 2 hr at room temperature in a humid atmosphere. These two solutions can be combined ahead of time and added together, or the antigen test solution can be added to the wells first, followed by the labeled antibody. However, the labeled antibody solution should not be added first.

   The amount of labeled antibody that is added will ultimately determine the signal strength; however, the antibody should not be added in excess over the binding capacity of the plate (see p. 573).

   All antibody and antigen dilutions should be done in 3% BSA/PBS with 0.02% sodium azide. Azide should not be included in the dilution buffer if horseradish peroxidase is used as the detection reagent.

10. Remove the unbound antibody and antigen by four washes with PBS.

Determine and quantitate the amount of labeled antibody bound to the plate (p. 591).

NOTES

i. If the amount of antigen bound to the plate is too low to produce a strong signal, the solid phase can be changed to nitrocellulose, which can bind approximately 1000 times more protein per surface area. Nitrocellulose can be purchased either as sheets or sealed to the bottom of microtiter wells. See p. 606 for special handling procedures.

ii. Any of blocking solutions on p. 496 can be substituted for the 3% BSA solution.

iii. Antigen-coated plates can be stored after the blocking step (step 7) either in PBS with 0.02% sodium azide at 4°C for 1 week or indefinitely at −20°C after removing the blocking solution.

iv. This assay may also be performed using an indirect detection method. The primary antibody is not labeled, but the remainder of the assay is performed as above. After the final wash (step 10), add a labeled secondary reagent. Incubate for 2 hr at room temperature, wash, and quantitate.

MAKING THE ASSAY QUANTITATIVE

To compare the levels of antigen in different samples, prepare serial dilutions of each antigen test solution in blocking buffer. Perform the remainder of the assay as above. To determine the relative amounts of antigen, compare the midpoints of the titration curves. To determine the absolute amount of antigen, compare these values with those obtained using known amounts of pure antigen in a standard curve.

For maximum sensitivity, first titrate the amount of standard antigen solution needed to coat the plate versus a fixed, high concentration of labeled antibody. Plot the values and select the lowest level that will yield a strong signal. Next, using plates coated with this amount of standard antigen solution, titrate the labeled antibody. Plot these values and select a level of antibody that is within the linear portion of the curve. Maximum range is obtained by choosing a point near the saturation level.

ANTIBODY CAPTURE ASSAYS—DETECTING AND QUANTITATING ANTIGENS USING ANTIGEN COMPETITION ASSAYS

An antibody capture assay performed in antibody excess can be used to determine the presence and level of antigens in test solutions. The test solution is allowed to bind directly to a solid phase, and any unbound proteins are removed by washing. Then an antibody specific for the antigen is added and allowed to bind. After unbound antibodies are removed by washing, the amount of antibody adhering to the solid phase is determined by using a labeled secondary reagent. This secondary reagent could be a labeled anti-immunoglobulin antibody, protein A, or protein G. These assays cannot be used if the antigen being studied is a rare component of the test solution. In this case, the specific binding is obscured by the background. A secondary technique such as immunoblotting may be particularly valuable to help distinguish the specific signal from the background when rare antigens are being studied.

The specificity of the antibody will determine the degree of purity needed to detect the antigen. Polyclonal sera will contain extraneous antibodies that will bind and score against some antigen preparations. Often these can be distinguished by titrating the sera. Because of their specificity, monoclonal antibodies or affinity-purified polyclonal antibodies can be used to analyze less pure sources of antigen.

Making these assays quantitative is difficult for samples that contain widely varying amounts of total protein. In these cases, the total amount of protein added to the solid phase must be standardized.

1. Prior to the assay, prepare the secondary reagent. Suitable secondary reagents include anti-immunoglobulin antibodies, protein A, or protein G. These reagents can be purchased from commercial sources with many different labels or they can be prepared as described on p. 319.

2. Nitrocellulose is the most suitable support for these assays, because its high binding capacity permits the detection of less abundant antigens in the sample. If using the nitrocellulose for a sheet assay, mark the nitrocellulose into 3-mm squares using a soft lead pencil. Cut the sheet to the proper size for the number of assays to be performed. If using the nitrocellulose paper in a dot blot apparatus, cut to the dimensions of the apparatus.

3. Antigens should be prepared in neutral pH buffers without additional proteins. If no other buffers are dictated by the experimental design, use PBS. Detergents should be avoided if possible. If they must be used, test the effects of different possible detergents on antigen binding to the nitrocellulose. For sheet assays, the antigen test solutions are added to the center of individual squares (1 μl/spot). Incubate for 30 min at room temperature in a humid atmosphere. For dot blot assays, pre-wet the sheet by floating on water for 5 min. Fit the sheet into the apparatus and apply the antigen test solution to the region of nitrocellulose that is exposed. Use 30 μl/well. Incubate for 2 hr at room temperature in a humid atmosphere.

4. If using a dot blot apparatus, remove the nitrocellulose sheet. Wash the nitrocellulose with two changes of PBS.

5. Block the remaining sites for protein binding on the nitrocellulose by incubating the sheet in 3% BSA/PBS with 0.02% sodium azide for at least 2 hr at room temperature.

6. Wash the nitrocellulose sheet twice in PBS.

7. Add primary antibody at a suitable dilution to the entire sheet. Use 1 ml/cm^2. Incubate with agitation for 2 hr at room temperature. The amount of antibody to be added can be determined in preliminary tests. Higher amounts will increase the sensitivity and extend the range of the assay, but also will increase the possibility of detecting nonspecific antigens.

   All antibody dilutions should be done in 3% BSA/PBS with 0.02% sodium azide.

8. Remove the unbound antibody by four washes with PBS.

9. Add the labeled secondary reagent. Use 1 ml/cm^2. The amount of labeled secondary reagent should be determined in preliminary tests. For accurate comparisons, the secondary reagent should be used in excess. For routine comparisons, the level of secondary reagent should be adjusted to produce a easily detected signal. All dilutions should be done in blocking buffer (3% BSA/PBS with 0.02% sodium azide).

   Incubate with agitation for 2 hr at room temperature.

10. Remove the unbound antibody by washing four times with PBS for 5 min each.

Determine and quantitate the amount of labeled secondary reagent bound to the sheet (p. 591).

NOTES

i. Any of blocking solutions on p. 496 can be substituted for the 3% BSA solution.

ii. If the antigen is impure, then a solid-phase substrate of higher capacity than nitrocellulose may be required to generate adequate signal strength. For this purpose, diazotized paper is useful as it can bind approximately 100 times more protein than nitrocellulose. With this variation, background problems caused by nonspecific binding or spurious cross-reactions may be severe.

iii. Directly labeled primary antibodies can be used in this test.

MAKING THE ASSAY QUANTITATIVE

To compare the levels of antigen in different samples, prepare serial dilutions of each antigen test solution in PBS. Perform the remainder of the assay as above. To determine the relative amounts of antigen, compare the midpoints of the titration curves. To determine the absolute amount of antigen, compare these values with those obtained using known amounts of pure antigen in a standard curve. To ensure that the assays are accurate, the amount of primary unlabeled antibody and secondary labeled reagent must be in excess. This can be determined by titrating the reagents, diluted in blocking buffer, on sheets coated with saturating amounts of the antigen.

OTHER APPLICATIONS

- **Column profiles** Assays of this design are extremely useful where rapid estimates of antigen presence and quantity are required for large sample numbers. A good example of this application is the detection of antigen-positive fractions from conventional chromatography columns or gradients.